Net type, tow duration and day/night sampling effects on the composition of marine zooplankton derived from metabarcoding

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Abstract

DNA metabarcoding requires only a single DNA fragment to detect a species in mixed zooplankton samples, compared to morphology-based methods that rely on the presence of intact specimens. However, metabarcoding protocols have not yet been fully standardised, thus hindering data comparability between studies. To converge on standardised metabarcoding protocols, we used an experimental field-sampling approach to compare the effects of sampling gear (ring-, Manta- and WP2 nets), day and night (DN) sampling and tow duration (5-, 10- and 15-minute tows) on marine zooplankton composition. High-throughput sequencing of the cytochrome c oxidase subunit I (COI) gene region with different primers and taxonomic assignment of amplicon sequence variants at 97% similarity to barcode records were used to identify species. Metabarcoding detected a total of 224 species, of which 92% matched prior occurrence records from the region. Malacostraca (crabs, hermit crabs, lobsters, prawns and shrimps) was the best-represented class (49%), followed by Copepoda (21%), Actinopterygii (ray-finned fishes; 21%), and Gastropoda (9%). Species counts ranged from 9–61 species per tow, with high proportions of unique species in replicate tows. Mean species counts did not differ significantly between net types, DN samples or tow durations, respectively. Proportionate representation amongst taxonomic classes remained within a narrow range, except when sampling deeper habitats with a smaller mesh size. DN samples showed no evidence of daily vertical migration of zooplankton. Consistent inferred species composition across net types, tow duration and DN sampling treatments reflects high detection sensitivity of individual-based sampling, allowing for greater flexibility in planning of zooplankton sampling regimes.

Key words: Experimental approach, gear-effects, high-throughput sequencing, species diversity, standardisation of field sampling

Introduction

DNA metabarcoding provides a rapid and accurate method to record the species composition of mixed marine zooplankton samples (Bucklin et al. 2016; Laakmann et al. 2020). It allows for high-resolution detection in biodiversity censuses (Questel et al. 2021), the ability to identify all life stages to the species level, including eggs, larvae and juveniles (Zhang et al. 2018; Govender

et al. 2019) and detection of rare species in large mixed samples (Govender et al. 2022a). In combination with traditional quantitative and morphological analysis of samples, metabarcoding offers a comprehensive tool to characterise zooplankton community structure and biomass (Hirai et al. 2021; Matthews et al. 2021).

Nevertheless, the direct integration of metabarcoding data into existing morphology-based time series remains challenging (Deagle et al. 2018). Limitations to the use of metabarcoding data are primer bias (Clarke et al. 2017) and that online reference databases that link taxonomic species descriptions to DNA barcodes remain incomplete and affected by ongoing taxonomic revisions (Ficetola et al. 2016; Zhang et al. 2018; Singh et al. 2021). Barcode records on reference databases vary in quality and accuracy, necessitating validation of identified species against occurrence records (Keck et al. 2023). Logistical challenges to the uptake of metabarcoding information in biomonitoring initiatives and marine environmental management include affordability, skilled manpower, standardised protocols and sustained dialogue amongst stakeholders (Aylagas et al. 2020; Huggett et al. 2022).

Furthermore, few standards exist for the collection of field data, laboratory approaches or analysis pipelines, thus hindering comparability of data collected by different laboratories (but see https://metazoogene.org/). Historical literature contains many studies on the effects of sampling methods and gear on quantitative estimates of zooplankton and species composition derived from morphological analysis. Examples are the performance of different plankton net types (Wiebe and Holland 1968; Gjøsæter et al. 2000), plankton nets versus plankton recorders (Stehle et al. 2007), duration of tows (Wiebe 1972), variation in swept area (Takasuka et al. 2019) and day versus night tows (Shaw and Robinson 1998; Suthers et al. 2006). In contrast, few studies exist on the effects of sampling strategy and gear on species composition derived from metabarcoding data. Hirai et al. (2015) found higher diversity when using molecular-, compared to morphological analysis of samples collected with different net types, confirming the sensitivity of metabarcoding for characterising zooplankton communities. Monchamp et al. (2022) found coherence in aquatic community composition derived from metabarcoding and classical species surveys, but differences persisted amongst the different workflows.

The standardisation of protocols for collecting metabarcoding data is a key aspect to consider when planning long-term biomonitoring surveys and for the integration of molecular information into existing quantitative time-series data. To propose standards, empirical evidence needs to be provided on best-practice approaches to field sampling. To do so, we used an experimental approach to compare the effects of sampling gear, day and night sampling and duration of tows on the metabarcoding-based composition of zooplankton samples collected in coastal waters off eastern South Africa. Specific aims were to: (1) generate a species list of zooplankton present in samples using metabarcoding; (2) validate identified species by cross-referencing with occurrence records; and for standardisation (3) evaluate the effects of different net types, day versus night sampling and tow duration on metabarcoding-based species composition.

Materials and methods

Experimental approach

Discrete sampling stations at 20 m, 100 m and 200 m depth soundings along a cross-shelf transect near Durban in the KwaZulu–Natal (KZN) Province of South Africa were sampled for zooplankton using plankton nets towed behind a boat (Suppl. material 1: table S1). Three net types were used: a ring net with a diameter of 0.8 m and 500 µm mesh; a Manta net with a rectangular frame of 0.5 × 0.15 m and 500 µm mesh; and a WP2 plankton net with a 0.55 m diameter and 200 µm mesh. Mechanical flow meters (Hydro-Bios; Model 438 110; Apparatebau GmbH) were attached centrally in the ring- and Manta net openings to determine the volume of water sampled during each tow. The water volume was calculated as the number of impeller revolutions multiplied by the pitch (0.3 m per revolution) and the area of the net opening. A Sea-Bird SB19plus Profiler CTD (Sea-Bird Scientific) was deployed at each sampling station to measure water temperature (°C), salinity (ppt), fluorescence and oxygen content (mg/l) at approximately 2 m below the sea surface.

Two field sampling protocols were followed to sample zooplankton: a net-type experiment to compare the effects of three different net types deployed during the day, using the same tow duration; and a day/night (DN) and tow duration experiment, using one net type (ring net) to compare the effects of day and night tows and of increasing tow durations, on zooplankton species composition derived from metabarcoding analysis.

For the net-type experiment, the ring- and Manta nets were towed horizontally just below the surface for 5 minutes at a time with a ground speed of 2–3 knots. The WP2 net was lowered to 10 m above the seafloor at each sampling station and then hauled vertically. All tows were undertaken at night, between sunset and sunrise. To provide more comprehensive data across a spatio-temporal range, samples were collected from stations at 100 and 200 m depth contours in September 2018 and August 2019. To avoid carry-over of DNA between successive tows, the ring net and cod-end were thoroughly rinsed with seawater between tows. To test for potential cross-contamination of samples (transfer of DNA and organisms between successive tows), three replicate ring net tows were performed at each sampling station. Even though the test cannot completely rule out cross-contamination, we hypothesised that high variability in the numbers of species identified in successive tows and a low level of shared species would indicate a low incidence of cross-contamination. A total of 20 samples were available for the net-type experiment, 12 from ring nets (four sets of three replicate tows) and four samples each from the Manta and WP2 nets.

For the DN/tow duration experiment, only the ring net was used, with tows conducted as above. Daytime samples were collected between noon and sunset and night samples from approximately 1 hour after sunset. Nets were towed for 5, 10 and 15 minutes at a time. Samples were collected from stations at 20 m and 100 m depth contours, in May and September 2022. A total of 24 samples were available for the DN/tow duration experiment, comprising of four samples in each of the six categories (day and night tows of 5, 10 and 15 minutes each).

All samples from both net-type and DN/tow duration experiments were washed from the tow net cod-ends into jars with 97% ethanol and stored at −20 °C before further processing in a genetics laboratory. Ethanol in sample jars were replaced after 24 hours to ensure optimal long-term storage conditions and to minimise the degradation of samples over time.

Laboratory processing of samples

Extraction of genomic DNA

Individual tow net samples were homogenised in the 97% ethanol solution for 45 s using a consumer blender (Defy PB7354X, 350Wand 22,000 rpm) (Govender et al. 2022b). Between samples, the blender was washed with a soapy solution to remove residual material, rinsed with a 10% bleach solution to degrade remaining DNA and rinsed again with 70% ethanol. Three subsamples per homogenate were taken to improve diversity estimates. Each subsample (10 ml of zooplankton) was centrifuged at 1200× g for 1 min, repeated to remove excess ethanol; thereafter, 40 mg of tissue was transferred to a sterile tube. The remaining DNA extraction process was carried out as described by Govender et al. (2023).

PCR amplification, library preparation and high-throughput DNA sequencing

Polymerase chain reactions (PCRs) were performed in triplicate to minimise stochastic effects, bias and amplification errors (Dopheide et al. 2019). Taxon-specific mini-barcode primer cocktails (n = 5 primer cocktails) targeting mainly Malacostraca (crabs, hermit crabs, lobsters, prawns and shrimps) and Actinopterygii (ray-finned fishes) and one universal primer set targeting metazoan diversity of the highest abundance were selected according to Govender et al. (2022b) (Suppl. material 1: table S2). PCRs (25 µl) contained 0.25 µl Q5 High-Fidelity DNA Polymerase (0.02 U µl⁻¹, New England BioLabs), 5 µl Q5 reaction buffer (1×), 5 µl Q5 high GC enhancer (1×), 0.5 µl dNTPs (10 mM of each), 1 µl forward and reverse primers (5 µM), 1 µl template DNA (10 ng µl⁻¹), 2 µl additional $MgCl_2$ (25 µM), 2 µl bovine serum albumin (BSA; 1 mg ml⁻¹) and nuclease-free water. Thermal cycling consisted of initial denaturation at 98 °C for 30 s and 25 cycles at 98 °C for 10 s each, annealing at 46 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 4 min. All PCRs included a no-template negative control (2 µl water). PCR products were visualised on a 1% (w/v) TBE agarose gel containing 0.02% ethidium bromide (EtBr). Amplicon size was determined using a 100-bp molecular weight marker (Solis Biodyne). The triplicate PCR products for each of the six primer sets were pooled and quantified using a Qubit 2.0 Fluorometer (Life Technologies, California, USA). The pooled products were consolidated into a single sample for each tow-net haul, to create 44 libraries (one per individual tow) with equimolar concentrations (5 ng/µl). The libraries were sequenced using the Illumina MiSeq platform (Illumina) at the KZN Research and Innovation Platform (KRISP, South Africa) using a MiSeq Nano Reagent Kit v.2 (500 cycles) and following the protocols outlined in Govender et al. (2022b).

Taxonomic assignment of Amplicon Sequence Variants (ASVs) and cross-referencing with occurrence records

The dada2 algorithm (Callahan et al. 2016) implemented in Qiime2 v. 2019.10 (Bolyen et al. 2019) was used for quality control checks, chimera removal, filtering, trimming of primers, truncation of forward and reverse reads and merging of paired-end reads into amplicon sequence variants (ASVs). The ASVs were queried on BOLD systems version 4 (https://www.boldsystems.org) and NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/; query cover > 80%) in January 2023. A 97% sequence identity threshold was used for taxonomic assignment to species level (see Govender et al. (2022a)). ASVs assigned to the same species were merged manually using MS Excel.

Species detected by metabarcoding were further validated by cross-referencing with occurrence records obtained from online databases such as the World Register of Marine Species (WoRMS; https://www.marinespecies.org), the Ocean Biodiversity Information System (OBIS; https://obis.org), the Global Biodiversity Information Facility (GBIF; https://www.gbif.org) and online literature.

Criteria used to compare metabarcoding-based species composition amongst experimental categories were individual species records, cumulative and mean (± standard deviation) species counts per experimental treatment and the proportional representation of Malacostraca, Actinopterygii, Copepoda (copepods) and Gastropoda (gastropods) in samples.

To examine variability at the level of individual sampling sites and test for potential cross-contamination, species collected by replicate ring net tows at individual sampling sites were compared. A null hypothesis of no difference in species counts was tested and Venn diagrams were constructed to compare unique and shared species within each set of three replicate tows. Where appropriate, Levene's test was used to test for equality amongst variance of samples, followed by single and two-factor ANOVA to test for differences amongst sample means.

Results

Physical measurements and water volume filtered

Water temperature (20.8–21.5 °C) and salinity (35.2–35.5 ppt) measured at 2 m below the sea surface remained within a narrow range across all sampling trips (Suppl. material 1: table S3). Dissolved oxygen ranged between 4.9 and 7.1 mg/l, indicating well-oxygenated surface water. Chlorophyll-a fluorescence values indicated similar phytoplankton concentrations in surface water at different sampling stations (e.g. 100 m and 200 m depth contours) of the same trip, but more variation between sampling trips undertaken in different years (e.g. 2018, 2019 and 2022). Overall, the environmental conditions during sampling trips were typical of the region and varied within a relatively narrow range.

For the net-type experiment, the average flow volumes for 5-minute tows ranged between 19.1 and 39.8 kl per replicate set of three ring nets and between 36.0 and 37.6 kl per tow for individual Manta nets (Suppl. material 1: table S4). For the DN experiment, similar volumes (average of all tows combined, irrespective

of their duration) were filtered by day (96.9 kl) and at night (92.1 kl). For the tow duration experiment, filtered water volumes (average per duration category) increased proportionally during 5-minute (47.2 kl), 10-minute (97.1 kl) and 15-minute tows (139.3 kl).

High-throughput sequencing results and species counts derived from metabarcoding

Sequencing was efficient with minimal filtering needed (for both forward and reverse reads) when merging the paired-end reads for all 44 zooplankton libraries (Table 1). For the 20 net-type libraries, a total of 2.3 million read counts were consolidated into 135,821 merged reads, of which a total of 830 sequences were available for analysis across all groups amplified. The 830 sequences were then collapsed into 128 ASVs that were matched to a species level with a > 97% sequence similarity to sequences on BOLD or GenBank. Of the 830 sequences, 329 (40%) sequences remained unassigned or could not be assigned to a species level at 97% similarity or above.

Table 1. High-throughput sequencing outputs for each sampling station and event.

|	Library	Depth (m)	Read count	Merged reads	Total amplicon sequence variants	Merged amplicon sequence variants assigned to species level (97%)
Net type experiment	2018	100	600746	21750	133	23
		200	464824	33241	263	74
	2019	100	619826	32631	292	65
		200	644274	48199	311	63
Total across sites	–	–	2329670	135821	830	128
Day/night/duration experiment	Trip 1	20	639068	19180	228	48
		100	641680	24187	456	107
	Trip 2	20	602386	17125	335	67
		100	647686	17117	348	78
Total across sites	–	–	2530820	77609	1047	178

For the 24 DN/tow duration libraries, a total of 2.5 million read counts were consolidated into 77,609 merged reads, of which a total of 1047 sequences were available for analysis across all groups amplified. The 1047 sequences were then collapsed into 178 ASVs that were matched to species level with a > 97% sequence similarity to sequences on BOLD or GenBank. Of the 1047 sequences, 589 (56%) sequences remained unassigned or could not be assigned to a species level at 97% similarity or above.

For all 44 tows combined, metabarcoding detected zooplankton belonging to 27 orders, 89 families, 160 genera and 224 species (Suppl. material 1: table S5). Malacostraca was the best-represented order (110 spp.), followed by Copepoda (48 spp.), Actinopterygii (46 spp.) and Gastropoda (20 spp.). Of the 224 species, 170 (76%) matched prior distribution records from South Africa, with a further 37 species (17%) not previously recorded from South Africa, but known from the broader Western Indian Ocean (WIO) region, of which KZN forms the south-western extreme. Seventeen identified species (8%) were out of range (no records

from South Africa or the WIO) and some species had no occurrence records on online databases – hence, they were categorised as false positives. In total, 207 species (92% of the total) matched distribution records from South Africa and/or the WIO, confirming that the 97% sequence similarity threshold used in the study was robust for zooplankton species identification using COI.

Analysis of replicate tow data

The number of species identified per individual tow ranged from 9 to 61, with the mean number of species (± SD) for each replicate set of three tows being 29.7 ± 27.2; 29.3 ± 11.0; 23.0 ± 5.0; and 13.3 ± 3.8 species. The hypothesis of no difference in the number of species caught in replicate ring net tows was rejected in two of the four cases (χ^2 = 8.205 and 49.92; df = 2; p < 0.05 in both cases), but accepted in the other two (χ^2 = 2.151 and 2.174; df = 2; p > 0.05 in both cases). Venn diagrams indicated high ratios of unique species as a proportion of all species identified within each replicate set of three tows (Fig. 1), i.e. 0.82; 0.54; 0.52; and 0.38, respectively. The smallest- and largest proportions of shared species amongst replicate tows were 0.18 and 0.62. High variability in the numbers of species captured by replicate tows, high levels of unique species and rejection of the null hypothesis reflect the patchiness of zooplankton communities sampled at individual sampling sites. The low level of shared species implies a low incidence of cross-contamination during sampling at sea.
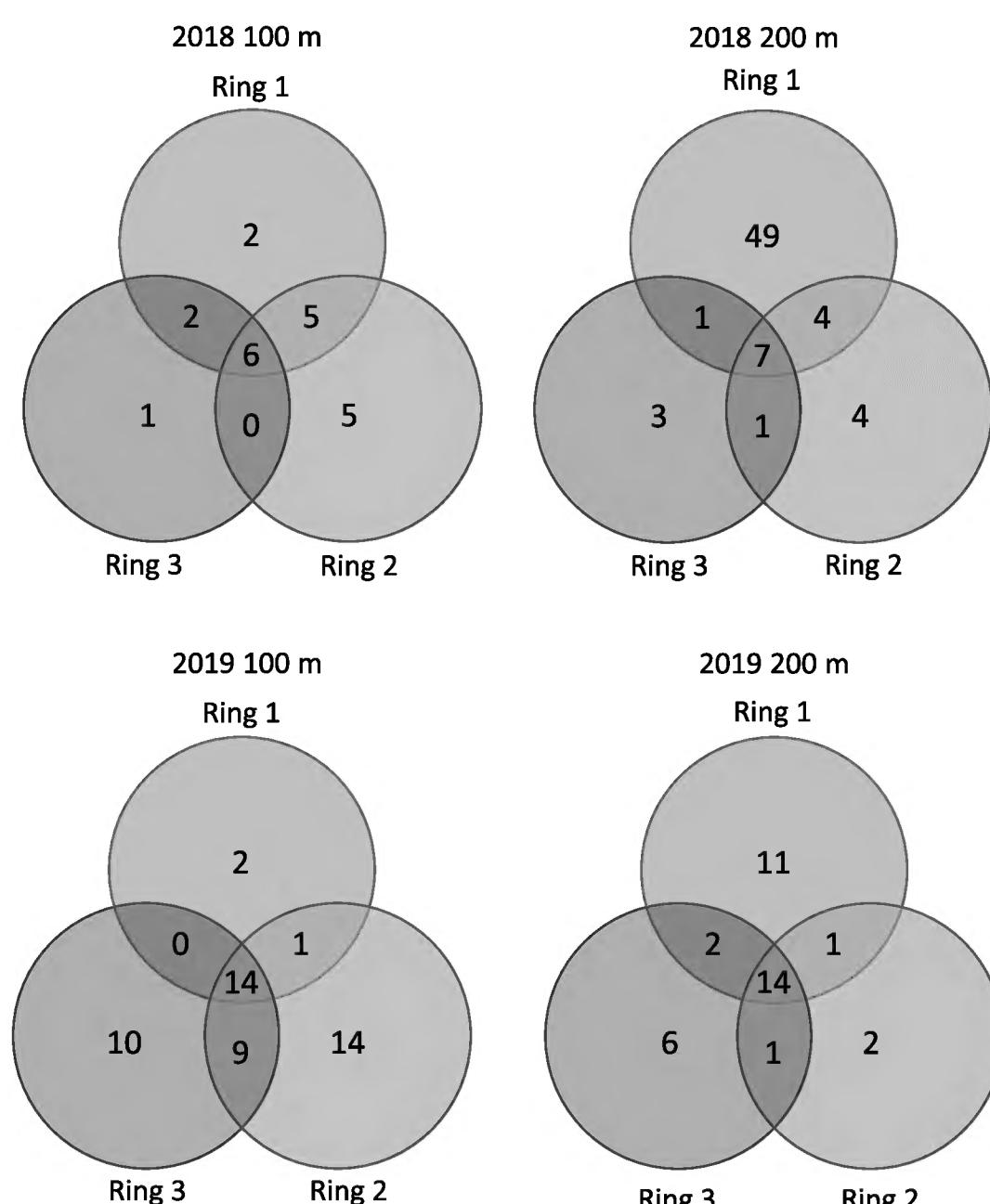
Figure 1. Venn diagrams of the numbers of species collected by four sets of three replicate ring net tows in 2018 and 2019, at 100 and 200 m depth contours. The diagrams show unique and shared species per tow.

Net-type effects

In the net-type experiment, metabarcoding identified 106 species from ring net samples (n = 12 tows), 47 from WP2 net samples (n = 4 tows) and 34 from Manta net samples (n = 4 tows). Mean species counts (±SD) for each net type were 23.8 ± 14.5 for ring nets, declining to 20.5 ± 10.4 for WP2 nets and 13.5 ± 8.7 for Manta nets (Fig. 2). Data were normally distributed and Levene's test confirmed the homogeneity of variances (F = 0.202; df = 2; p = 0.819). A single-factor ANOVA found no significant difference in the mean number of species collected per net type (F = 0.952; df = 2; p = 0.406), although the sample size (number of nets towed) was small.

On average, Malacostraca contributed the largest proportion of all species identified from ring-(50.4%) and Manta net samples (48.2%), but in WP2 nets, there were more Copepoda (39.0%) than Malacostraca (34.2%) (Fig. 3). Actinopterygii contributed 17.3 to 20.9% of identified species, irrespective of net type and Gastropoda contributed 3.7 to 6.1% of species.

DN / tow duration effects

In the DN/tow duration experiment, metabarcoding could identify 125 species from ring net samples collected during the daytime and 116 species from night samples, but the difference was not significant (χ^2 = 0.336; df = 1; p = 0.562) (Fig. 4A). For tow duration, there was no significant difference in species counts in pooled samples for 5-minute (102 species), 10-minute (109 species) and 15-minute (101 species) tows (χ^2 = 0.365; df = 2, p = 0.83) (Fig. 4B).

Mean species counts per tow (all taxa combined) did not differ significantly between day (27.8 ± 7.7) and night samples (25.4 ± 11.7) (Students t-test; p = 0.56) and nor did counts differ between 5-minute (26.0 ± 10.9), 10-minute (27.1 ± 6.0) and 15-minute tows (26.6 ± 12.6) (ANOVA, F = 0.0244, df = 2, p = 0.976). The simultaneous analysis of the effects of DN and tow duration using a two-factor ANOVA with equal replication accepted all null hypotheses of no difference between DN (F = 0.289; p = 0.598) and tow-duration (F = 0.0224; p = 0.978) combinations, with no evidence for interaction between factors (F = 0.521; p = 0.602).
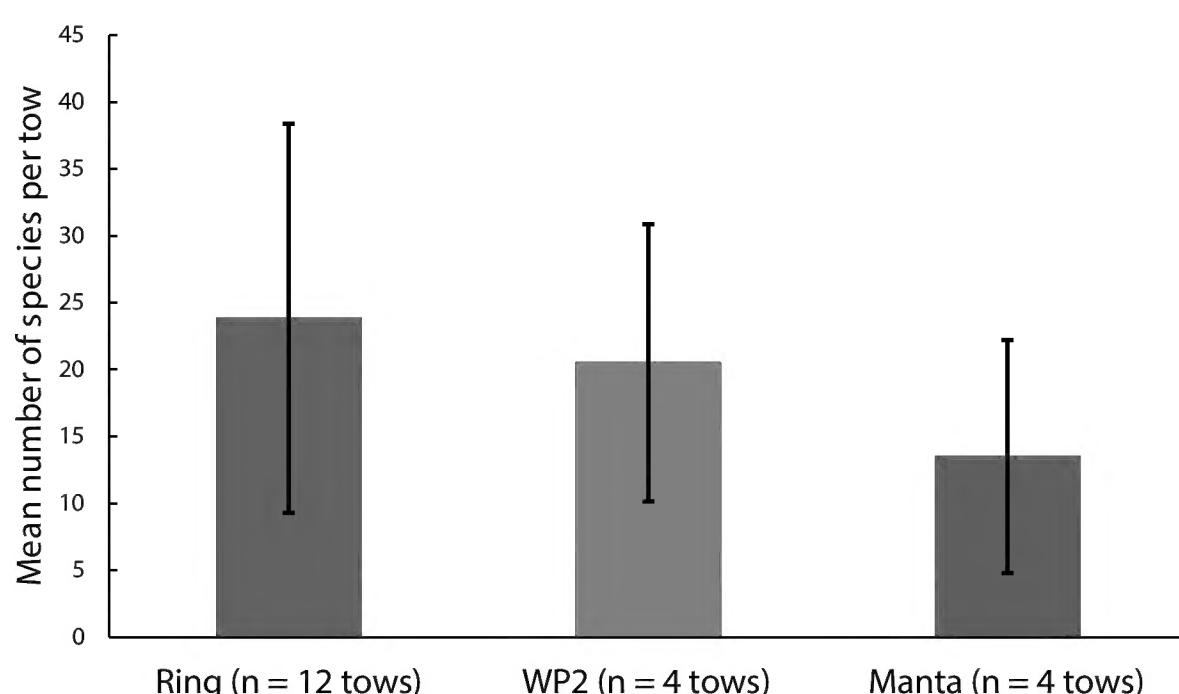
**Figure 2.** Mean number of species per tow (± standard deviation) identified from samples per net type.

Ring Net

5%

17%

51%

27%

■ Malacostraca ■ Copepoda
■ Actinopterygii ■ Gastropoda

WP2 Net

6%

21%

34%

39%

■ Malacostraca ■ Copepoda
■ Actinopterygii ■ Gastropoda

Manta Net

4%

18%

48%

30%

■ Malacostraca ■ Copepoda
■ Actinopterygii ■ Gastropoda
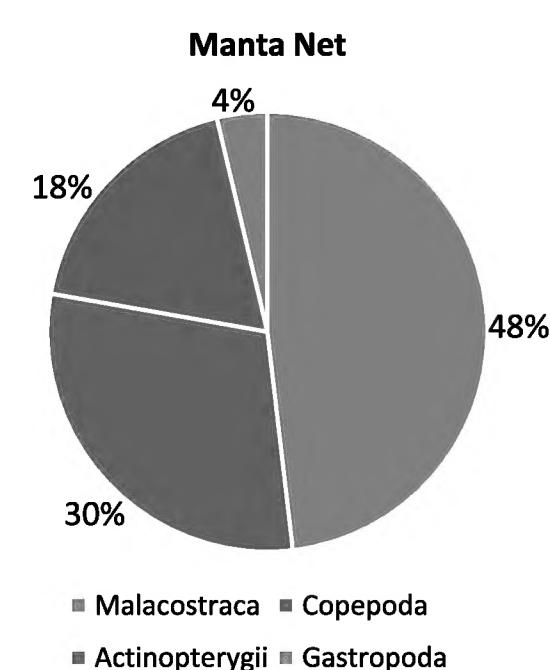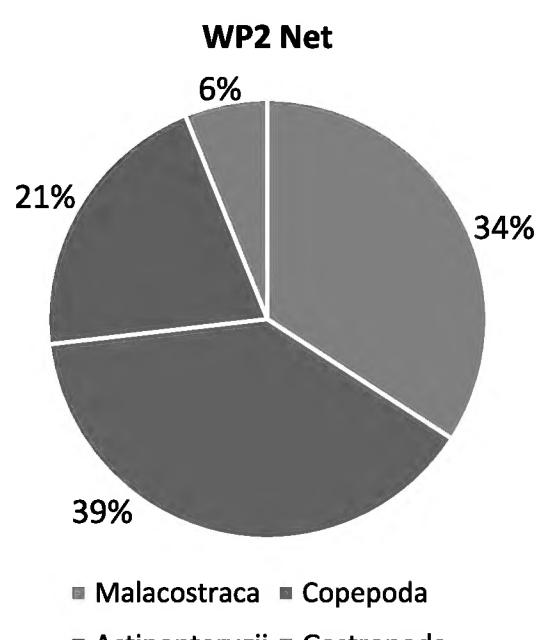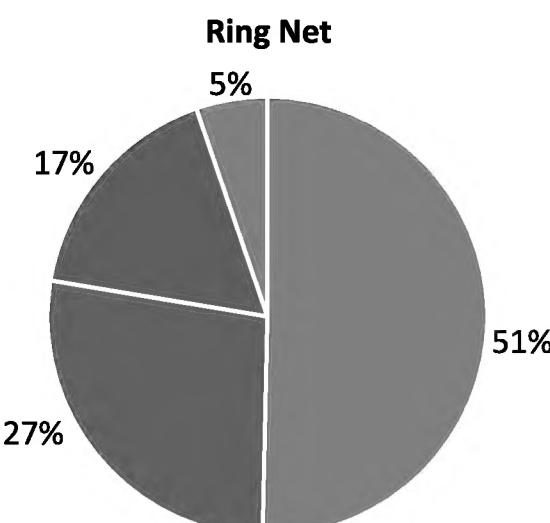
Figure 3. Number of species identified per taxonomic class as a proportion of all species per net type sampled.

Malacostraca made up the bulk of species in both day (45.6% of species identified) and night samples (44.3%), followed by Copepoda (23.7% and 33.4%), Actinopterygii (17.1% and 14.4%) and Gastropoda (13.5% and 7.9%) (Fig. 4A). Proportionally more copepod species were present in night than day samples, but ray-finned fishes and gastropods were more species rich in day samples. Tow duration did not affect the proportionate representation of species in samples, which remained within a narrow range for each group, irrespective of tow duration (Fig. 4B).

A

B
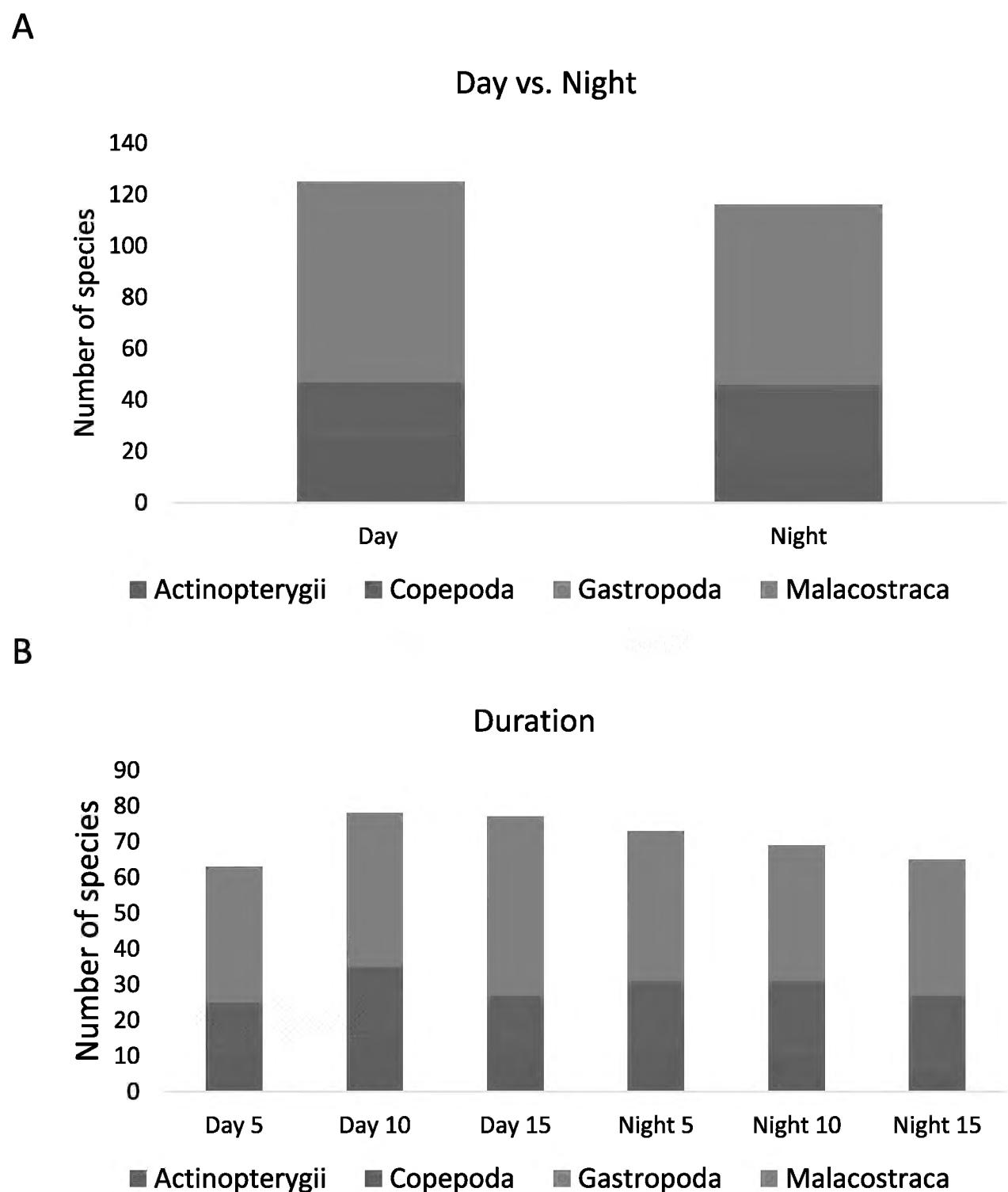
Figure 4. Number of species identified per taxonomic class as a proportion of all species for (A) day and night sampling and (B) tow duration (5, 10, 15 minutes) during day and night sampling (e.g. Day 5 = 5-minute tow during daytime).

Discussion

The purpose of this study was to examine the effects of field sampling strategy (net-type, tow duration, day or night sampling) on the species composition of marine zooplankton derived from metabarcoding. Field sampling effects on the outputs of metabarcoding studies in the marine environment are rarely discussed in literature, as most methodological discussions focus on the molecular aspects of analyses. Our study contributes to the standardisation of methods used in metabarcoding studies of zooplankton, with a longer-term objective of comparability amongst datasets and integrating metabarcoding data into existing morphology-based time-series trends.

A total of 224 zooplankton species with > 97% similarity to barcode reference sequences were identified during the metabarcoding analysis. This is an underestimate of the true species richness in the samples. Some 40% of the sequences for the net-type experiment and 56% of the DN/tow experiment sequences could not be assigned to a species level because they did not match records on BOLD or GenBank with > 97% similarity. Furthermore, the mini-barcode primers used in our study were taxon-specific for Malacostraca

and Actinopterygii (Govender et al. 2019, 2022a), thus under-sampling other zooplankton groups. An additional universal mini-barcode primer (Folmer et al. 1994; Leray et al. 2013) succeeded in amplifying copepods and gastropods, but the success rates for these taxa are unknown. No other groups (e.g. amphipods, chaetognaths, hydrozoa) had sequence similarities of > 97% and they were, therefore, excluded from the analysis, even though individual specimens were observed in the mixed samples.

Seventeen of 224 species (8%) identified from barcode reference data appeared to be false positives, with > 97% sequence similarity, but no validatory occurrence records from South Africa or the WIO region. False positive identifications can result from inaccuracies on BOLD and GenBank reference databases (Genis-Armero et al. 2023; Keck et al. 2023) or a lack of divergence in the COI gene region (used on BOLD) of closely-related species (Ershova et al. 2023). Alternatively, the putative false-positive species identified with metabarcoding may be present in the region, but not yet reported on the occurrence records that were examined. We suggest that this is a common theme in metabarcoding analyses of pelagic ecosystems, where drifting larvae are dispersed widely by ocean currents (Vereshchaka et al. 2016; Govender et al. 2022c) or where species introductions occur because of human activities (e.g. transported in ships' ballast water) (Briski et al. 2012).

Selecting similar conditions for sampling across experiments (e.g. common sampling sites, replicate sampling, completing the full array of experimental treatments during the same trip) was aimed towards reducing natural variability so that the effects of net type, DN and tow duration on metabarcoding-based composition would be enhanced. A low level of shared species and high variability were observed between replicate ring net tows, suggesting a minimal occurrence of cross-contamination at sea. No significant differences were found in the mean numbers of species identified from sampling with various net types (ring, WP2 and Manta nets). Ring- and Manta nets were towed at depths < 5 m and, therefore, sampled similar epipelagic habitats during the same sampling trip. These two net-types caught similar proportions of malacostraca, copepods, ray-finned fishes and gastropods. WP2 nets were hauled vertically, from a maximum depth of 190 m and caught a greater proportion of copepods and fewer malacostraca than the ring and Manta nets, a difference attributed to deeper habitats sampled and/or a smaller mesh size used (200 µm) in the WP2 net.

The selectivity of zooplankton tow nets can, *inter alia*, be influenced by avoidance behaviour, clogging of the net mesh, escape and patchiness (Pillar 1984; Gjøsæter et al. 2000; Skjoldal et al. 2013). We used the same mesh size (500 µm) on ring- and Manta nets and did not observe clogging of the mesh. The two nets have different shapes (round versus rectangular) and net openings ($0.5\,m^2$ versus $0.075\,m^2$), implying differences in selectivity and increased avoidance with the smaller net (Wiebe and Holland 1968; Gjøsæter et al. 2000; Sameoto et al. 2000). Flow meter readings for the two net types were not considered comparable, because the Manta net was towed at the sea surface, where readings would have been affected by turbulence. Overall, the ring net caught more species (on average) than the Manta net, although the difference was not statistically significant. The small sample size (12 ring- and four Manta net tows) and high variability in species counts between individual tows increased the likelihood of a type-2 error in the statistical analysis.

The DN samples showed no evidence of daily vertical migration (DVM) of zooplankton (reviewed by Bandara et al. (2021)). No increase in the number of species collected in surface waters (i.e. in ring- and Manta net tows) were observed at night, when zooplankton migrate to surface layers to feed, compared to the day, when they migrate deeper to avoid predation. In contrast, there were marginally more species observed in day samples, although not significant. Plausible explanations for the absence of DVM in DN samples were that depths sampled were too shallow (20 and 100 m bottom depth) for measurable DVM observations or that night tows should have been undertaken later at night, to allow deeper zooplankton layers more time to rise to the surface. Alternatively, we suggest that individual-based sampling (as in metabarcoding) will be less sensitive to DVM, compared to population-based quantitative studies, because not all individuals in a population are expected to participate in DVM. Hence, some individuals of a given species are expected to remain in surface waters during daytime and detectable in metabarcoding data, when the bulk of the population migrates deeper.

The absence of a significant difference in metabarcoding-based species counts in tows with increasing duration can partially be explained by the effects of zooplankton patchiness in the water column. Zooplankton patchiness refers to the aggregation of zooplankton in specific areas (or patches) at horizontal and vertical planes, on scales ranging from centimetres to kilometres (George 1981). Patchiness constitutes a crucial component of zooplankton sampling error (Wiebe and Holland 1968). In morphology-based studies, longer tow durations increased the number of species that could be identified, because it increased the likelihood of sampling different patches (Wiebe 1970, 1971; King and Robertson 1978). Individual patches may comprise of species or life stages that cannot be identified morphologically. In contrast, the metabarcoding-based species counts did not increase significantly in tows longer than 5 minutes, thus confirming the much greater detection sensitivity of molecular methods. Only short tows are, therefore, needed to obtain representative presence/absence data.

In conclusion, the detection of 224 zooplankton species, of which 92% matched prior distribution records, emphasises the robustness of metabarcoding in characterising zooplankton communities. Mean species counts obtained from metabarcoding analysis did not differ significantly between net types, DN samples or tow duration, respectively. The proportionate representation amongst taxonomic classes remained within a narrow range across experimental treatments, except when sampling deeper habitats with a smaller mesh size. The consistency of metabarcoding-based species composition across experimental treatments reflects high detection sensitivity of individual-based sampling, allowing for greater flexibility in planning of zooplankton sampling regimes. Thus, we contribute important empirical evidence for standardising field sampling methods when collecting marine zooplankton to be analysed with metabarcoding.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: AG, JCG, SPS, SWM. Data curation: AG, JCG. Formal analysis: JCG, AG. Funding acquisition: JCG. Investigation: AG, JCG. Methodology: SWM, AG, JCG, SPS. Project administration: JCG. Resources: JCG. Software: AG. Supervision: JCG, SPS, SWM. Validation: AG, JCG. Visualization: JCG, AG. Writing - original draft: JCG, AG. Writing - review and editing: SPS, SWM.

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Data availability

All raw sequence reads and codes used to perform analyses are available from Figshare (https://doi.org/10.6084/m9.figshare.25577301.v2); additionally, the raw sequence data have been uploaded to NCBI (https://www.ncbi.nlm.nih.gov/sra/PRJNA1115141).

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Supplementary material 1

Supplementary information

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Data type: docx
Explanation note: **table S1**. Latitudinal and longitudinal data points for sampling stations at 20 m, 100 m and 200 m depth soundings along a cross-shelf transect near Durban in the KwaZulu–Natal (KZN) Province of South Africa. **table S2.** The six primer cocktails used in this DNA metabarcoding study (first round PCR): each of the COI primer cocktails amplify different fragments of the COI-5P gene region. Illumina adapter target sequences (indicated in bold and underlined) were used in accordance with the workflow from the Illumina 16S Metagenomics protocol. These adapter targets allow Nextera indexing and Illumina adapter addition through PCR. **table S3.** Environmental data measured with the Sea-Bird SB19plus Profiler CTD at approximately 2 m below the sea surface. Data are unavailable for the 1st part of the DN/tow duration experiment. **table S4.** Water volume (kilolitres, kl) passing through ring nets (0.5 m² net opening) and manta nets (0.075 m² net opening) based on flow meter data. Data are unavailable for the 1st part of the DN/tow duration experiment. **table S5.** Species detected by metabarcoding of zooplankton collected over the continental shelf of Durban, South Africa and verification of adult distribution ranges.

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